

Proliferating Cell Nuclear Antigen (PCNA) Is Required for Cell Cycle-regulated Silent Chromatin on Replicated and Nonreplicated Genes^{*[5]}

Received for publication, July 21, 2010, and in revised form, September 2, 2010. Published, JBC Papers in Press, September 2, 2010, DOI 10.1074/jbc.M110.166918

Andrew Miller^{‡§}, Jiji Chen^{§¶}, Taichi E. Takasuka^{‡§}, Jennifer L. Jacobi^{‡§}, Paul D. Kaufman^{||}, Joseph M. K. Irudayaraj^{§¶}, and Ann L. Kirchmaier^{‡§¶1}

From the [‡]Department of Biochemistry, the [¶]Department of Agricultural and Biological Engineering, and the [§]Purdue University Center for Cancer Research, Purdue University, West Lafayette, Indiana 47907 and the ^{||}Program in Gene Function and Gene Expression, University of Massachusetts Medical School, Worcester, Massachusetts 01605

In *Saccharomyces cerevisiae*, silent chromatin is formed at *HMR* upon the passage through S phase, yet neither the initiation of DNA replication at silencers nor the passage of a replication fork through *HMR* is required for silencing. Paradoxically, mutations in the DNA replication processivity factor, *POL30*, disrupt silencing despite this lack of requirement for DNA replication in the establishment of silencing. We tested whether *pol30* mutants could establish silencing at either replicated or non-replicated *HMR* loci during S phase and found that *pol30* mutants were defective in establishing silencing at *HMR* regardless of its replication status. Although previous studies tie the silencing defect of *pol30* mutants to the chromatin assembly factors Asf1p and CAF-1, we found *pol30* mutants did not exhibit a gross defect in packaging *HMR* into chromatin. Rather, the *pol30* mutants exhibited defects in histone modifications linked to *ASF1* and CAF-1-dependent pathways, including SAS-I- and Rtt109p-dependent acetylation events at H4-K16 and H3-K9 (plus H3-K56; Miller, A., Yang, B., Foster, T., and Kirchmaier, A. L. (2008) *Genetics* 179, 793–809). Additional experiments using FLIM-FRET revealed that Pol30p interacted with SAS-I and Rtt109p in the nuclei of living cells. However, these interactions were disrupted in *pol30* mutants with defects linked to *ASF1*- and CAF-1-dependent pathways. Together, these results imply that Pol30p affects epigenetic processes by influencing the composition of chromosomal histone modifications.

To regulate expression of a gene epigenetically, cells must efficiently assemble specialized chromatin at those genes dur-

ing or shortly after DNA replication each cell cycle. This process ensures that the gene expression state is inherited in future generations. In the budding yeast *Saccharomyces cerevisiae*, an epigenetic process called silencing prevents transcription of the mating-type genes at the silent mating-type loci *HMR* and *HML* (1). When silent chromatin is first formed, the silent information regulator proteins Sir1p, Sir2p, Sir3p, and Sir4p are recruited to the *HM* loci through their physical interactions with proteins bound to DNA elements called silencers adjacent to the *HM* loci. At *HMR*, the four Sir proteins interact with each other and with the origin recognition complex (ORC),² Rap1p and Abf1p bound to the *E* silencer (2–9). Sir2p, Sir3p, and Sir4p then spread along the chromosome and across the mating-type genes *a2* and *a1* at *HMR* as the deacetylase Sir2p removes acetyl groups from histones H3 and H4 and creates binding sites for Sir3p and Sir4p on nucleosomes (7, 9) (see also Ref. 5). Once silent chromatin is formed upon passage through S phase, the *a2* and *a1* genes at *HMR* are inactivated and their silenced state will be inherited as the genome is duplicated in subsequent generations (1, 10–14).

Although DNA replication itself is not required to establish silent chromatin in S phase (10–12), a link between DNA replication and silencing in *S. cerevisiae* has been reinforced by numerous studies. In yeast, many mutated or misexpressed replication-related genes affect silencing, including those encoding proteins involved in initiating DNA replication, in leading and lagging strand DNA synthesis, and in deposition of histones onto newly synthesized DNA (15–21). In this study, we explored how PCNA, an evolutionarily conserved protein central to each of these events during DNA replication, contributes to the formation of silent chromatin.

PCNA, which is encoded by the yeast *POL30* gene, serves as an accessory factor for DNA polymerases δ and ϵ and acts by tethering these polymerases to their template DNA during DNA replication, thus enhancing their processivity (22–24). During DNA replication, PCNA is loaded as a trimer around DNA forming a homotrimeric, sliding, ring-shaped clamp (25, 26). RF-C is the primary clamp loader complex that mediates the association of PCNA with DNA (25, 27). In addition, PCNA

* This work was supported, in whole or in part, by National Institutes of Health Grants R01 GM55712 (to P. D. K.) and GM31105 (to J. Rine, with whom A. L. K. worked at the time). This work was also supported by Kimmel Scholar Award SKF-03-010 (to A. L. K.), a National Science Foundation award (to A. L. K.), a grant from the Purdue Office of the Vice President of Research (to A. L. K. and J. M. K. I.), and an American Cancer Society institutional research grant to the Purdue University Center for Cancer Research. Support for initial experiments was provided by the American Cancer Society (PF-01-126-01-MBC to A. L. K.).

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental "Experimental Procedures," "Results," "References," Tables 1–6, and Figs. 1–5.

¹ To whom correspondence should be addressed: Dept. of Biochemistry and Purdue University Center for Cancer Research, Purdue University, 175 S. University St., West Lafayette, IN 47907. Tel.: 765-494-0977; Fax: 765-494-7897; E-mail: kirchmaier@purdue.edu.

² The abbreviations used are: ORC, origin recognition complex; PCNA, proliferating cell nuclear antigen; CAF, chromatin assembly factor; Sir, silent information regulator; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; FRT, flippase recognition target.

tethers several other proteins to DNA, including Rad27p (FEN1 in mammals) and Cdc9p (DNA ligase I), which together process and ligate newly synthesized Okazaki fragments during lagging strand synthesis (28, 29) (for review see Refs. 30 and 31). PCNA also binds the chromatin assembly factor complex CAF-1 (20, 32–34). CAF-1 consists of three subunits (Cac1p, Cac2p, and Cac3p), acts as a chaperone for newly synthesized histones H3 and H4, contributes to replication- and repair-coupled nucleosome assembly (34–36), and can act synergistically with a second chromatin assembly factor, Asf1p, to achieve this feat (37, 38). Several of these *POL30*-interacting factors also affect silencing when mutated or misexpressed (17–19).

The varying phenotypes of *pol30* mutants (19, 20, 38–40) indicate that PCNA can influence silencing and other cellular processes by multiple mechanisms. The silencing defects of several *POL30* mutants, including *pol30-8*, *pol30-6*, and *pol30-79*, have been linked to CAF-1- or *ASF1*-dependent pathways (20, 38). Each of these *pol30* mutants has defects in binding the large subunit of the CAF-1 chromatin assembly factor complex, Cac1p (20). Additionally, although no direct interaction between Asf1p and Pol30p has yet been reported, Asf1p can bind to the clamp loader RF-C (41) as well as to Cac2p (32, 37, 42) and can stimulate CAF-1-dependent chromatin assembly (37, 38, 43). In addition, the mammalian Asf1 interacts with histones and the putative replicative helicase Mcm2–7 (44).

PCNA influences the construction of heritable chromatin structures in multiple organisms. Mutation of the PCNA ortholog *mus209* suppresses position-effect variegation in *Drosophila* (45), and PCNA is linked to both DNA- and histone-modifying enzymes associated with epigenetic processes in mammals. Mammalian PCNA binds to and stimulates the activity of DNMT1, a DNA methyltransferase that maintains heritable methylation patterns on CpG islands (46–49). *In vitro*, PCNA and DNMT1 interact with the histone deacetylase HDAC1, which also functions in transcriptional silencing, and these proteins co-localize *in vivo* (46, 50, 51). PCNA also recruits human CAF-1 to DNA and serves as a mark for CAF-dependent chromatin assembly *in vitro* (33, 34). CAF-1, in turn, targets the methyl CpG-binding protein MBD1 and the H3-K9-specific methyltransferase SETDB1 to replication foci. When combined with DNA methylation, MBD1 and SETDB1 promote stable transcriptional silencing (30, 52). In addition, the mammalian H4-K20-specific methyltransferase SET8 binds to PCNA (53). Methylation of H4-K20, in turn, has also been associated with heterochromatin in multiple organisms (Ref. 54 and references within). Thus, PCNA plays a central role in epigenetic processes through recruiting chromatin-modifying machinery to replication foci.

Together, these observations compelled us to explore the role of *POL30* in silencing and to test whether DNA replication was required for *pol30*-dependent silencing defects in yeast. Surprisingly, *pol30* mutants were defective in establishing silencing at *HMR*, regardless of its replication status upon passage through S phase. Single molecule analyses (fluorescence lifetime imaging microscopy and fluorescence resonance energy transfer (FLIM-FRET)) revealed that PCNA interacted with the acetyltransferases Rtt109p and SAS-I *in vivo* but *pol30*p mutants had defects in these interactions. Rtt109p- and

SAS-I-dependent histone modifications were also reduced in chromatin isolated from *pol30* mutants, implying that histone acetylation defects linked to Asf1p- and CAF-dependent pathways influence silencing in these mutants.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains were generated by standard genetic techniques including homologous recombination, one-step gene replacement, and plasmid shuffling (55–58). Genotypes of parental strains are described in [supplemental Table 1](#), and plasmids used in this study are described in [supplemental Table 2](#).

Plasmid pAK876 was derived from pAK196 (11), which contains an EcoRI-HindIII fragment of a modified *HMR* locus containing a synthetic silencer with four Gal4p binding sites, a Rap1p binding site, and an Abf1p binding site in place of the *HMR-E* silencer and the genes encoding *a2* and *a1* but lacking the *HMR-I* silencer. This modified *HMR* is flanked by two FRT sites oriented to permit excision of a 2.6-kb covalently closed circular double-stranded DNA molecule from chromosome III in the presence of FLP recombinase. In pAK876, a PstI-XmaI fragment within this modified *HMR* that contained the *a2-a1* promoter region was replaced with 266 bp of heterologous DNA amplified from the 5' region of *bla* from pUC19 using oALK441 5'-CATGCCTGCAGGTCGACTCTGTCGTGTA-GATAACTACGATAC-3' and oALK442 5'-TCGGTACCCG-GGGATCCTCTCGACGAGCGTGACACCACGATG-3'. Excision of this modified *HMR* from chromosome III by FLP recombinase will also generate a 2.6-kb covalently closed circular double-stranded DNA molecule. Plasmid pAK928 (H3 K14R/H4) was derived from pWZ-414-F13 (59) by site-directed mutagenesis according to the QuikChange site-directed mutagenesis kit protocol (Stratagene, La Jolla, CA) using oligonucleotides oALK613 5'-AAATCCACTGGTGGTAGAGCC-CCAAGAAAACAAT-3' and oALK614 5'-ATTGTTTTCTT-GGGGCTCTACCACCAGTGGATTT-3'; plasmid pAK929 (H3 K14R/H4 K16R) was derived from pWZ-414-F24 (59) using the same oligonucleotide pair. Plasmid pAK995 (H3 K9Q/H4) was derived from pPK189 as described above using oligonucleotides oALK753 5'-CAAACAGCTAGACAATCC-ACTGGTGGTAAAGCCC-3' and oALK754 5'-GGGCT-TTACCACCAGTGGATTGTCTAGCTGTTTG-3'. Plasmid pAK1008 (H3 K9Q K56R/H4) was derived from pAK995 as described above using previously described oligonucleotides oALK691 and oALK692 (40). Plasmids pAK1004 (H3 K9R,K56Q/H4) and pAK1005 (H3 K9R,K56R/H4) were derived from pAK873 (H3 K9R/H4) as described above using previously described oligonucleotides oALK691 and oALK692 or oALK642 and oALK643, respectively (40).

Plasmids pAK1105 and pAK1106 were constructed by amplifying *POL30-GFP* from yAK5110 using oALK1038 5'-CTGCAGCCCGGGGATCCACTAGTTCTAGAGCGG-CCGCCAATGCTACACGTGCTTGA-3' and oALK1040 5'-GAATTTCGATATCAAGCTTATCGATACCGTCGACAC-TAGTAGGTCTTAGTGTGACTGTCA-3'. PCR products were digested with HindIII and BamHI and inserted into HindIII-BamHI of pRS415 or with SpeI and inserted into SpeI of pRS416. Plasmids expressing *pol30-CFP* mutants were gen-

POL30 and the Establishment of Silencing

erated from pAK1105 by site-directed mutagenesis according to the QuikChange site-directed mutagenesis kit protocol. pAK1107 (*pol30-6-CFP*) was made using oALK1026 5'-CAA-GCTGCTGCTGCCTCAAGAGTTC-3' and oALK1027 5'-GAACTCTTGAGGCAGCGACAGCTTG-3'; pAK1108 (*pol30-79-CFP*) using oALK1030 5'-GATGCTGATTTTCGCAAAGGCTGAAGAATTACAG-3' and oALK1031 5'-CTGTAATTC-TTCAGCCTTTGCGAAATCAGCATC-3'; and pAK1126 (*pol30-8-CFP*) using oALK 1114 5'-TTCCAAGAATATGCATGTGCCCATCCTGTTACG-3' and oALK 1115 5'-CGT-AACAGGATGGGCACATGCATATTCTTGAA-3'.

Strains encoding various combinations of Pol30-CFPp, Rtt109-YFPp, Sas4-YFPp, Sas5-YFPp, Trx3-CFPp, and Spc29-YFPp fusion proteins were generated by homologous recombination using PCR products generated from pKT212 or pKT211 (60) plus oligonucleotides complimentary to the 3'-ends of the specified loci (sequences available upon request) and by standard genetic crosses.

Cell Cycle Experiments—Cell cycle experiments were conducted as described previously (11, 61). In these experiments, silencing was regulated by expressing Gal4-Sir1p from the methionine-regulated promoter *MET3*. Cells were grown in synthetic complete medium lacking histidine and tryptophan and containing 2% glucose and methionine to repress Gal4-Sir1p expression. Cells were then collected by centrifugation, resuspended in synthetic complete medium lacking histidine and tryptophan and containing 65 μ M methionine and 2% raffinose with 1 or 10 μ g/ml α -factor, and incubated at 30 °C for approximately 3 h until >90% of the cells arrested in G₁ phase and formed shmoos. To induce FLP recombinase to excise the *HMR* locus, galactose was added to the medium to a final concentration of 2% and cells were incubated for 1 to 1.5 h at 30 °C. Alternatively, to leave the *HMR* locus in the chromosome, cells were incubated further in medium containing 2% raffinose. Cells were concentrated onto 0.45- μ m nitrocellulose filters (Whatman), washed with synthetic complete medium lacking histidine and tryptophan and containing 2% raffinose, resuspended in synthetic complete medium lacking histidine, tryptophan, and methionine and containing 2% raffinose plus 1 or 10 μ g/ml α -factor, and incubated for 1 h at 30 °C to express Gal4-Sir1p in G₁. Cells were then concentrated by filtration as described above, washed with synthetic complete medium lacking histidine, tryptophan, and methionine and containing 2% glucose, resuspended in synthetic complete medium lacking histidine, tryptophan, and methionine and containing 2% glucose plus 10 μ g/ml Pronase, 30 μ g/ml benomyl, and 10 μ g/ml nocodazole, and incubated at 30 °C for 2 h. Samples were harvested at each time point for analysis of cell cycle arrests by microscopy or flow cytometry, and analysis of RNA to monitor silencing of *a1* at the *HMR* locus and of DNA to monitor excision of *HMR* from chromosome III was conducted as described previously (11, 55, 61). Statistical analyses for cell cycle and topology assays were conducted using the Wilcoxon rank sum test with MSTAT v2.6.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation experiments were performed using two independent yeast strains for each genotype and analyzed by real-time PCR on an ABI Prism 7000 as described previously (61, 62) using

anti-Pol30p (41, 63), anti-Sir2p, and anti-Sir3p antibodies (9) or anti-H3 antibodies (Abcam, catalog No. ab1791). Cells were grown in synthetic complete medium containing 2% glucose and lacking histidine or histidine and methionine. Oligonucleotides used for chromatin immunoprecipitation analyses at *MAT* and *HMR* have been described previously (40, 61, 62), and sequences are available upon request.

RNA Analyses—Total RNA was isolated from logarithmically growing cells, *a1* mRNA levels relative to control *SCR1* levels were analyzed by RNA blot assays or quantitative real-time PCR as described previously. Oligonucleotide sequences used to generate *a1* and *SCR1* probes and to conduct quantitative real-time PCR are available upon request (11, 61, 62, 64).

Confocal Fluorescence Lifetime Imaging Analysis—Yeast were grown logarithmically in synthetic complete medium with 2% glucose to an A_{600} of 0.8 prior to FLIM-FRET analysis. FLIM measurements (65) were performed on single living cells ($n = 100$ – 200) with a confocal setup using an inverted Olympus IX71 microscope (Center Valley, PA) equipped with a picosecond 40-MHz pulsed 467-nm diode laser (Microtime 200, PicoQuant GmbH, Berlin) for excitation at 3–10 microwatts of power (66). The laser beam was focused on the sample using an apochromatic 60 \times water immersion objective (1.2 N.A.); the emitted fluorescence was collected by the same objective and separated from the excitation beam by a dichroic mirror using appropriate band-pass filters (Omega Optical, Brattleboro, VT). Single-photon avalanche photodiodes (SPAD; SPCM-AQR, PerkinElmer Life Sciences) were used to record the emitted photons, and fluorescence was measured using the time-correlated single photon counting (TCSPC) module in the time-tagged time-resolved (TTTR) mode (Time Harp200, PicoQuant). To obtain fluorescence lifetimes, TCSPC decay curves were fitted by a double exponential function using SymphoTime software (version, 5.13, PicoQuant). The donor lifetimes were calculated in the absence and presence of the acceptor and FRET efficiency (E) and distance (R) between two energy-transferring molecules were then obtained from

$$E = 1 - \frac{\tau_{DA}}{\tau_D}, R = \left(\frac{1 - E}{E} \right)^{1/6} R_0 \quad (\text{Eq. 1})$$

where τ_{DA} and τ_D are the donor excited state lifetime in the absence and presence of acceptor, respectively. R_0 is the Förster distance (the distance at which 50% energy transfer between donor and acceptor exists). For CFP and YFP, this distance is 52 Å (67).

Extraction of Chromatin-associated Histones—Histones were isolated from the chromatin fraction of nuclei derived from logarithmic yeast cultures as described previously (40).

Protein Blot Analyses—Chromatin fractions or whole cell extracts from each strain were separated on 15% SDS-polyacrylamide gels and transferred to PVDF membranes (BioRad). Membranes were blocked with a 1:1 dilution of Odyssey blocking buffer (Li-Cor Biosciences, catalog No. 927-40000) and 1 \times phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 5.4 mM NaH₂PO₄, and 1.47 mM KH₂PO₄). For analysis of chromatin-associated histones, protein blots were probed with anti-acetylhistone H3 (Lys-9) antibodies (1:1000; Cell Signaling

Technology, catalog No. 9671) or anti-Acetyl-Histone H4 (Lys-16) antibodies (1:5000; Upstate, catalog No. 07-329) using Alexa Fluor anti-rabbit IgG as the secondary antibody (1:2000 for analysis of H3-K9ac or 1:10,000 for analysis of H4-K16ac; Molecular Probes, catalog No. A21109). Membranes were stripped with 0.2 M NaOH at room temperature and reprobed with anti-histone H3 antibodies (1:30,000; Abcam, catalog No. ab1791) using Alexa Fluor anti-rabbit IgG as the secondary antibody (1:40,000). For analysis of whole cell extracts, protein blots were probed as above except with dilutions of 1:20,000 for anti-PCNA antibodies (41, 63) and 1:20,000 for anti-histone H3 antibodies. Antibodies were diluted in 50% Odyssey blocking buffer, 0.5× PBS, and 0.1% Tween 20 prior to use. Blots were analyzed using an Odyssey infrared imager and Odyssey software v1.2 according to manufacturer's instructions (Li-Cor Biosciences, Odyssey User Guide, version 1.2). Statistical analyses for quantitative protein blots were conducted using the Wilcoxon rank sum test.

Colony Color and Mating Assays—Colony color assays, conducted using two independent yeast strains for each genotype, were performed as outlined in Fig. 4 and Refs. 40 and 68. Briefly, logarithmically growing yeast containing *ADE2* integrated between the *E* and *I* silencers at *HMR* were plated at a density of ~300 cells/plate on rich medium (YPD) plates, incubated at 30 °C for 2 days, and stored at 4 °C for approximately 3 days prior to collecting images with a Leica MZ125 microscope and SPOT 4.1.1 imaging software. In this assay, red colonies indicated that *HMR::ADE2* was silenced, white colonies indicated *HMR::ADE2* was expressed, pink colonies indicated a defect in maintaining or inheriting silencing, and sectoring colonies indicated a defect in establishing silencing at *HMR::ADE2*. Patch mating assays were conducted with two independent clones for each genotype as described in Fig. 5. Quantitative mating assays were performed in triplicate as described in Table 7 and supplemental Table 6 (40, 62, 68). Mating indicates that the *HMR* locus was silenced.

RESULTS

***pol30* Mutants Have Defects in Establishing Silencing on Non-replicated Templates**—Although DNA replication is not required to establish silencing (10–12) (see also Refs. 14, 69, and 70), mutations in *POL30* paradoxically lead to silencing defects (20, 38). It was possible that *pol30* mutants prevented the establishment of silencing in a replication-dependent manner (*i.e.* through disruption of Sir association at *HMR* during passage of a replication fork). Alternatively, *pol30* mutants may have had a defect in a replication-coupled process that resulted in a cellular state incompatible with silencing-replicated as well as non-replicated genes. Therefore, we asked whether silencing at a non-replicated *HMR* could be established in *pol30* cells as opposed to a replicated *HMR*. To conduct these experiments, we introduced *POL30* or *pol30-6*, *pol30-8*, or *pol30-79* mutations (71, 72) into yeast containing a regulatable *HMR* locus (10, 11) (Fig. 1A). This *HMR* contains a synthetic *E* silencer with four Gal4p binding sites, a Rap1p and an Abf1p binding site (*HMR-GalSS*), and the *a1* and *a2* genes, but it lacks the *I* silencer. Sir protein association and silencing at this *HMR* can be regulated by expressing Gal4-Sir1p via the methionine-re-

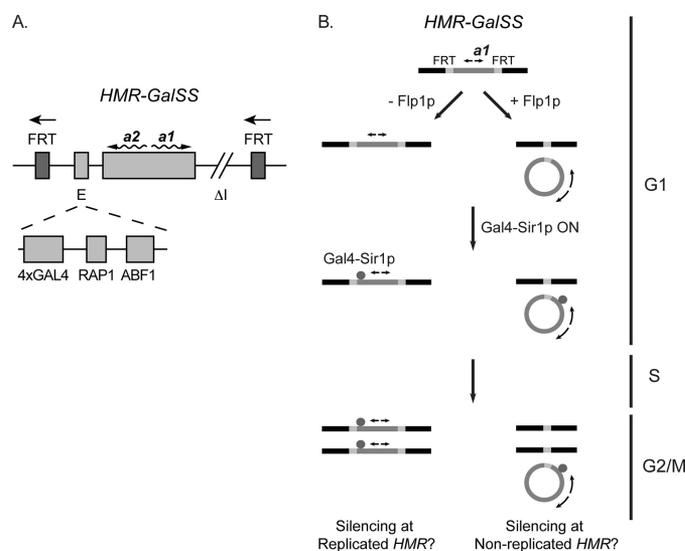


FIGURE 1. *pol30* mutants are defective in silencing a regulatable *HMR* locus. *A*, a regulatable *HMR* locus. The *HMR* contained a modified *E* silencer, *HMR-GalSS*, which comprised four Gal4 binding sites in place of the ORC binding site plus a Rap1p and an Abf1p binding site and the *a1* and *a2* genes, but lacked the *I* silencer. Expression of the chimeric Gal4-Sir1p via the *MET3*-repressible promoter in these cells enabled Sir protein association at *HMR*. Upon induction of Flp1p from the *GAL10* promoter, Flp1p binds to the FRT sites that flank *HMR* and excises *HMR* from chromosome III as a 2.6-kb double-stranded circular DNA molecule. *B*, experimental strategy. Cells grown in methionine lacking Gal4-Sir1p and expressed *a1* mRNA. Cells were arrested in G₁ with α -factor. Flp1p expression was then induced with galactose to excise *HMR* from the chromosome in G₁. Next, expression of Flp1p was repressed, and Gal4-Sir1p was induced in G₁ in medium containing raffinose and lacking methionine. Finally, cells expressing Gal4-Sir1p were released from G₁ and allowed to progress through the cell cycle until G₂/M where they were re-arrested with benomyl plus nocodazole.

TABLE 1
***pol30* mutants are defective in silencing**

Gal4-Sir1p	Relative efficiency of <i>a1</i> mRNA expression ^a			
	<i>POL30</i>	<i>pol30-8</i>	<i>pol30-6</i>	<i>pol30-79</i>
–	1	1	1	1
+	0.053 ± 0.036	0.096 ± 0.0022	0.36 ± 0.066	0.17 ± 0.015

^a Silencing at *HMR* was monitored in logarithmic *POL30* and *pol30* cells. For each strain, the relative level of *a1* mRNA was calculated as the ratio of PhosphorImager units as follows: [(*a1*/*SCR1*) in the absence or presence of Gal4-Sir1p]/[(*a1*/*SCR1*) in the absence of Gal4-Sir1p]. The data have been normalized to the ratios observed in the absence of Gal4-Sir1p to combine data from independent experiments: mean ± S.D., *n* = 3.

pressible promoter *MET3* (9–11, 61). This regulatable *HMR* is also flanked by FRT binding sites for the Flp1p recombinase and can be excised from the chromosome upon inducing *FLP1* via the *GAL10* promoter (11, 61). When excised from the chromosome, this *HMR*, which lacks its own origin of DNA replication, is not replicated upon passage through S phase, yet it can still be silenced in a Sir protein-dependent manner by the time cells reach G₂/M (11).

Initially, to confirm *POL30* mutants-affected silencing at this modified *HMR*, we monitored silencing in *pol30* versus *POL30* cells by measuring *a1* mRNA expression in the presence or absence of Gal4-Sir1p (Table 1). This analysis indicated that *pol30* mutants were partially derepressed at *HMR* relative to *POL30* cells, which is in support of previous observations (20, 38). We then evaluated the role of PCNA in silent chromatin formation by monitoring the establishment of silencing in *POL30*, *pol30-8*, and *pol30-79* cells in the presence and absence

POL30 and the Establishment of Silencing

TABLE 2
pol30 mutants are defective in establishing silencing in the absence of DNA replication

Arrest	<i>HMR</i> ^a	Relative efficiency of <i>a1</i> mRNA expression ^b		
		<i>POL30</i>	<i>pol30-8</i>	<i>pol30-79</i>
G ₁	Not replicated	1	1	1
G ₂ /M	Not replicated	0.16 ± 0.092	0.32 ± 0.075	0.49 ± 0.17
G ₁	Replicated	1	1	1
G ₂ /M	Replicated	0.15 ± 0.031	0.41 ± 0.20	0.36 ± 0.13

^a*HMR* was either excised in G₁ (Not replicated) or left within the chromosome (Replicated).

^b Establishment of silencing was monitored in *POL30* and *pol30* cells. For each strain, the relative level of *a1* mRNA expressed in G₁ and in G₂/M was normalized to an internal control transcript, *SCR1*. The data have been normalized to the ratios observed during the G₁ arrest, and the levels in G₂/M are expressed relative to those in G₁ to combine data from independent experiments: mean ± S.D., *n* = 3 (11).

of a replication fork at the modified *HMR* shown in Fig. 1A (Table 2). *pol30-6* mutants were not used for these cell cycle analyses because of their more severe growth defects (20, 38, 71). As outlined in Fig. 1B, *POL30*, *pol30-8*, or *pol30-79* cells lacking Gal4-Sir1p, and thus expressing *a1* from *HMR*, were arrested in G₁ with α -factor. Each initial culture was divided in two, and Flp1p was induced in one of the cultures to excise *HMR*; then Gal4-Sir1p was induced in both G₁-arrested cultures. Cells were then released from G₁ into S phase and rearrested at G₂/M with benomyl and nocodazole. Steady state levels of *a1* mRNA were used to measure the transcriptional status of *HMR* because *a1* mRNA has a short half-life (10–14, 61, 73) (Table 2), excision of *HMR* was confirmed by DNA blots, and cell cycle arrests were confirmed by microscopy or flow cytometry (data not shown). To monitor the establishment of silencing in each strain, the level of *a1* mRNA during the G₂/M arrest was compared with that observed during the G₁ arrest by RNA blot analyses (Table 2). *POL30* cells established silencing at the replicated chromosomal *HMR* upon passage through S phase, as observed previously (11, 61). In contrast, *pol30-8* and *pol30-79* cells were defective in establishing silencing at the chromosomal *HMR* relative to *POL30* cells ($p = 0.025$ for each comparison, $n = 3$). Similarly, *POL30* cells established silencing at the non-replicated extrachromosomal *HMR* upon passage through S phase (see also Ref. 11, 61), whereas both *pol30-8* and *pol30-79* cells were also defective in establishing silencing at the extrachromosomal *HMR* relative to *POL30* cells ($p = 0.025$ for each comparison, $n = 3$). Analogous negative control experiments using medium containing methionine to repress Gal4-Sir1p expression throughout the time course indicated that similar levels of *a1* mRNA were expressed in G₂/M relative to G₁ in the absence of Gal4-Sir1p (data not shown). These results indicated that *pol30-8* and *pol30-79* mutants were defective in establishing silencing in both the presence and absence of DNA replication through *HMR*. Moreover, as this modified *HMR* lacked an ORC binding site, PCNA was required under conditions in which ORC and replication initiation were not. This defect in establishing silencing could have been caused by a defect that occurred either in *cis* at *HMR* or in *trans* throughout the yeast nucleus or genome.

Lack of Evidence for Marking *HMR* with *POL30*—One possible explanation for the defects in establishing silencing in the *pol30* mutants is that Pol30p influences silencing through a replication-independent function of PCNA at *HMR* during the

cell cycle in which silencing was established. Consistent with this idea, PCNA that is left on DNA after replication in a SV40 DNA replication system *in vitro* can recruit CAF-1 to that DNA for chromatin assembly (33). Further, PCNA co-purifies with the elongator complex *in vitro* and *in vivo* (74), suggesting that a variety of chromosomal processes other than DNA synthesis can be affected by the presence of PCNA.

We reasoned if Pol30p had been preferentially left at the *HMR* locus during the S phase prior to the one in which we monitored the establishment of silencing and then remained at *HMR*, or if Pol30p simply preferentially associated with *HMR* in general, Pol30p would be enriched at *HMR* relative to other loci before its excision from chromosome III. To test this possibility, we performed ChIP analyses with anti-Pol30p antibodies (63) to test whether Pol30p was preferentially enriched at *HMR* in G₁-arrested cells. In these ChIP assays, Pol30p was not preferentially bound to *HMR* relative to *MAT* in G₁ either in the absence of Gal4-Sir1p or upon expression of Gal4-Sir1p in G₁ (supplemental Fig. 1). We had shown previously that this anti-Pol30p antibody can detect the association of Pol30p at stalled replication forks by ChIP (41, 75), and control experiments indicated PCNA was expressed efficiently in both *POL30* and *pol30* cells (supplemental Fig. 2). Thus, Pol30p was not preferentially localized to *HMR* in a Sir-independent or -dependent manner prior to the establishment of silencing. We therefore tested whether other aspects of chromatin composition were altered in the mutants.

Effects of *pol30* Mutants on the Topology of *HMR* and SIR Association at *HMR*—The above observations raised the possibility that both the replicated and the unreplicated *HMR* loci contained a preexisting *pol30*-dependent defect that prevented efficient silent chromatin formation during S phase. In this scenario, this *pol30*-dependent defect at *HMR* could have occurred during chromosomal replication and packaging of newly replicated DNA in the previous cell cycle, been maintained throughout G₂, M, and the next G₁ phase, and then interfered with silent chromatin formation in the following S phase in which the establishment of silent chromatin had been monitored.

We reasoned that the cause of the silencing defect in the *pol30* mutants could be related to the nature of the nucleosomes in these cells that were deposited during replication-coupled chromatin assembly. Consistent with this notion, the silencing-defective *pol30* mutants also have defects in interacting with nucleosome assembly factors (20, 38, 39), and cells lacking *CAC1* are reported to have reduced levels of histone H3 at *HMR* and other genomic loci (76). Thus, reduced histone deposition at *HMR* or an altered characteristic of the chromosomal histones in the *pol30* mutants may have contributed to their silencing defects. To assess the influence of *pol30* mutations on the chromatin structure of *HMR*, we examined the topological distributions of an excised circular *HMR* locus that lacked the *a1-a2* promoter, *HMR* $\Delta p266$, in order to avoid monitoring transcription-dependent effects on topology simultaneously. However, this analysis indicated that *HMR* was efficiently packaged in both *POL30* and *pol30* cells (See supplemental “Results” and supplemental Fig. 3).

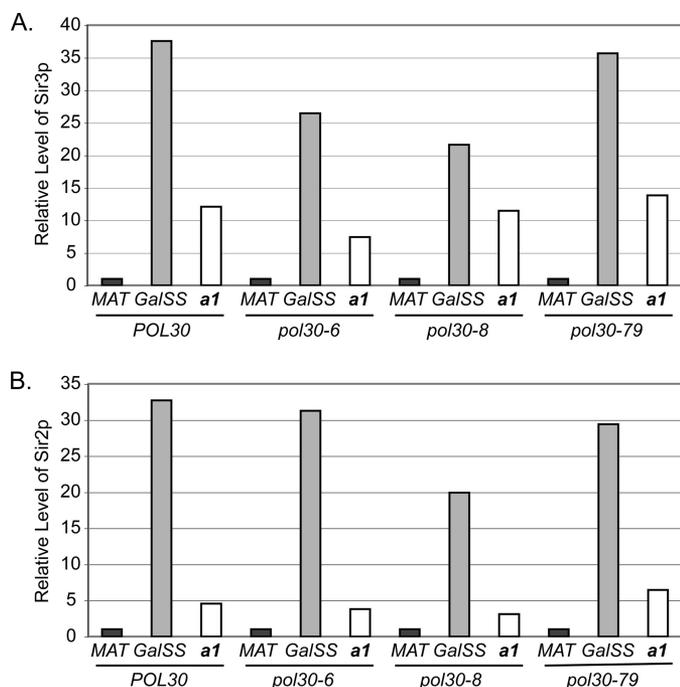


FIGURE 2. **Sir protein association at HMR in *POL30* and *pol30* cells.** Sir3 (A) and Sir2 (B) protein association at MAT (negative control locus), the synthetic silencer *GalSS*, and *a1* at HMR was monitored in the indicated strains by chromatin immunoprecipitation using anti-Sir3p or anti-Sir2p antibodies. Co-precipitating DNA was examined by quantitative real-time PCR (see “Experimental Procedures”). The efficiency of co-precipitation of each locus in each strain was normalized and expressed relative to MAT, with $MAT = 1$; $2^{[(Sir_{CT} - IgG_{CT})_{MAT} - (Sir_{CT} - IgG_{CT})_{locus}]}$. An average of $n = 2$ is shown. Data for individual replicates are provided in supplemental Tables 3 and 4.

To determine whether *pol30* mutants had defects in Sir protein association with HMR, despite their efficient packaging of HMR into chromatin, we conducted chromatin immunoprecipitation (ChIP) experiments to monitor Sir2p and Sir3p binding at HMR-*GalSS* in logarithmic *POL30* and *pol30* cells. In this analysis, Sir3p association at HMR-*GalSS* was slightly reduced in *pol30-8* and *pol30-6* mutants relative to *POL30* cells, and Sir2p levels were also lower in *pol30-8* relative to *POL30* cells (Fig. 2, A and B, respectively, and supplemental Tables 3 and 4). Although some Sir levels at HMR are altered by some *pol30* alleles, changes in Sir2p and Sir3p levels cannot explain the silencing defects of *pol30-79* mutants. Therefore, aspects other than Sir association must contribute to silencing defects, at least in *pol30-79* cells. Together, these results implied that, despite having defects in CAF-1 and *ASF1*-dependent pathways, *pol30* mutants could efficiently package DNA into nucleosomes, but stable Sir association with HMR was compromised in these mutants. Therefore, we explored whether other aspects of nucleosomes led to the defects in silencing in *pol30* mutants (see below).

***pol30* Mutants Have Defects in Histone Acetylation**—The above observations implied that, rather than gross defects in nucleosome density or marking HMR with Pol30p, other differences such as global defects in histone modifications coupled to DNA replication may account for the silencing defects of the *pol30* mutants. Global loss of histone modifications can lead to Sir relocalization throughout the genome and silencing defects (e.g. Refs. 77–79).

TABLE 3
Acetylation of Lys on chromatin-associated histone H3

Strain	Relative levels of H3-K9ac ^a
<i>POL30</i>	100
<i>pol30-8</i>	46 ± 2.8
<i>pol30-6</i>	87 ± 19
<i>pol30-79</i>	96 ± 50
<i>cac1Δ</i>	39 ± 3.3
<i>asf1Δ</i>	17 ± 2.0
<i>cac1Δasf1Δ</i>	14 ± 5.0
<i>cdc44-5</i>	75 ± 18
<i>sas2Δ</i>	100 ± 22

^a The level of chromatin-associated H3-K9ac relative to chromatin-associated H3 in each strain was determined by quantitative Western blot analysis as described under “Experimental Procedures” and was expressed as a percentage of that observed in *POL30* cells. Data were calculated as $(H3-K9ac/H3)_{mutant}/(H3-K9ac/H3)_{POL30} \times 100$, where mutant = indicated strain, mean ± S.D., $n = 3$ to 6 (except $n = 2$ for *sas2Δ*).

TABLE 4
Acetylation of Lys-16 on chromatin-associated histone H4

Strain	Relative levels of H4-K16ac ^a
<i>POL30</i>	100
<i>pol30-8</i>	69 ± 9.5
<i>pol30-6</i>	42 ± 3.4
<i>pol30-79</i>	67 ± 13
<i>cac1Δ</i>	59 ± 12
<i>asf1Δ</i>	70 ± 13
<i>cac1Δasf1Δ</i>	66 ± 16
<i>cdc44-5</i>	65 ± 24
<i>sas2Δ</i>	20 ± 3.1

^a The level of chromatin-associated H4-K16ac relative to chromatin-associated H3 in each strain was determined by quantitative Western blot analysis as described under “Experimental Procedures” and was expressed as a percentage of that observed in *POL30* cells. Data were calculated as $[(H4-K16ac/H3)_{mutant}/(H4-K16ac/H3)_{POL30}] \times 100$, where mutant = indicated strain, mean ± S.D., $n = 3$ (except $n = 2$ for *sas2Δ*).

To determine whether *pol30* mutants had defects in histone modifications, we examined acetylation of Lys-9 and Lys-56 on histone H3 and Lys-16 on histone H4 in whole cell extracts and in chromatin isolated from *POL30* and *pol30* cells. Like silencing in *pol30* mutants (20, 38), each of these histone modifications has been linked previously to *CAC1*- and *ASF1*-dependent pathways. Acetylation of Lys-9 on histone H3 occurs on newly synthesized histone H3, is cell cycle-regulated, and peaks in S phase in an *ASF1*-dependent manner (80–83). H3-K9ac is mediated by the acetyltransferases Rtt109p and Gcn5p (82, 84, 85). Acetylation of Lys-56 on histone H3 by Rtt109p also peaks during S phase and requires *ASF1* (75, 86–93). Also, acetylation of Lys-16 on histone H4 is mediated in part by the acetyltransferase complex SAS-I (94, 95), which interacts physically with both CAF-1 and Asf1p (96–98). Cells lacking the SAS-I subunits encoded by *SAS2*, *SAS4*, and *SAS5* have silencing phenotypes that overlap with those of *cac1* and *asf1* mutants (96, 97). We identified defects in histone H3-K56 acetylation in *pol30* mutants using this approach and have described these findings separately (40). Below, we describe our analysis of acetylation of H3-K9 and H4-K16 in the *pol30* mutants.

To determine whether *pol30* mutants with defects in *ASF1*- and *CAC1*-dependent pathways were defective in acetylating Lys-9 on histone H3 or Lys-16 on histone H4, we conducted quantitative protein blot analyses of chromatin-associated histones isolated from *POL30* and *pol30* cells (Tables 3 and 4 and supplemental Fig. 4). These analyses indicated that H3-K9 was hypoacetylated in chromatin isolated from *pol30-8* and *pol30-6* mutants as well as from *asf1Δ*, *cac1Δ*, and *asf1Δcac1Δ* mutants

POL30 and the Establishment of Silencing

($p = 0.061$ for *pol30-6*, $p = 0.018$ for other mutants relative to wild type, $n = 3$). Differences in H3-K9ac levels in *pol30-79* relative to *POL30* cells varied greatly and may reflect semi-stable epigenetic differences in H3-K9ac from culture to culture. Consistent with this notion, H3 K9R mutants exist in multiple epigenetic states (see below and Fig. 4B). The reduction in H3-K9ac was severe in *asf1Δ* and *cac1Δasf1Δ* mutants, implying that Asf1p played a key role in incorporating H3-K9ac into chromatin (see also Refs. 83 and 85). H3-K9ac was also reduced in *cdc44-5* mutants relative to wild-type cells. *CDC44* encodes the largest subunit of the PCNA-loading complex, RF-C (99), which helps recruit Asf1p to the replication fork (41). In contrast, wild-type levels of H3-K9ac were observed in cells lacking *SAS2*, indicating that SAS-I was not required for acetylation of this residue (Table 3; see also Ref. 98). Together these results implied both CAF-1 and Asf1p contributed to incorporation of H3-K9ac into chromatin during replication-coupled processes. Quantitative Western blot analyses also revealed that chromatin-associated histone H4 was hypoacetylated at Lys-16 in all *pol30* mutants relative to *POL30* cells ($p = 0.018$, $n = 3$) and H4-K16ac levels were reduced the other mutants as well (Table 4). Thus, chromatin-associated histones were hypoacetylated at multiple residues in *pol30* mutants (see also Ref. 40).

Rtt109p and SAS-I Interact with PCNA—To evaluate interactions between Pol30p and Rtt109p or the SAS-I complex *in vivo*, we turned to an approach based on FLIM-FRET between fusion proteins containing either CFP as the donor or YFP as the acceptor fluorophore. We assessed FRET donor lifetime changes of Pol30-CFPp in single living yeast cells also expressing Rtt109-YFPp, Sas4-YFPp, or Sas5-YFPp. Reduction in the CFP (donor) fluorescence lifetimes due to FRET with YFP was observed by recording the lifetime in yeast expressing Pol30-CFPp plus Rtt109-YFPp, Sas4-YFPp, or Sas5-YFPp relative to yeast expressing Pol30-CFPp only (Fig. 3A and supplemental Fig. 5A). No interactions were observed in cells expressing Pol30-CFPp plus the control protein Spc29-YFPp or the control protein Trx3-CFPp plus Rtt109-YFPp (supplemental Fig. 5A). A shift in peak position of lifetime distribution was observed in yeast expressing Pol30-CFPp plus Rtt109-YFPp, Sas4-YFPp, or Sas5-YFPp compared with Pol30-CFPp only (Fig. 3B and supplemental Fig. 5B). This shorter lifetime of the donor (Pol30-CFPp) in the presence of the acceptor fluorophore (Rtt109-YFPp, Sas4-YFPp, or Sas5-YFPp) indicates the close association of PCNA with Rtt109p and the SAS-I complex *in vivo*.

To confirm complex formation in the nuclei of single living cells, FRET efficiency between Pol30-CFPp and Rtt109-YFPp, Sas4-YFPp, or Sas5-YFPp was calculated by lifetimes obtained from the TCSPC decay histograms and fitted by a double exponential function. The lifetime of CFP, the calculated FRET efficiency, and the intermolecular distances are shown in Table 5 and supplemental Table 5. The FRET efficiency of Pol30-CFPp with Rtt109-YFPp, Sas4-YFPp, and Sas5-YFPp was ~28.6, 25.7, and 23.1%, respectively, which corresponds to a Förster distance of 6.1, 6.2, and 6.4 nm, respectively (which is less than the diameter of a nucleosome), between these pairs of proteins in living cells.

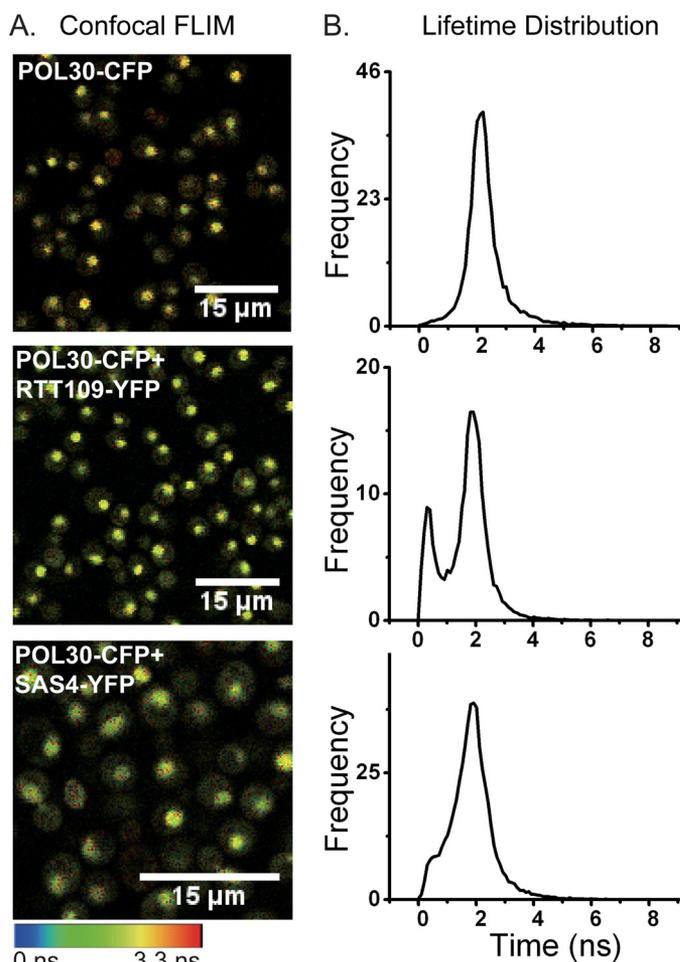


FIGURE 3. PCNA interacts with Rtt109p and SAS-I *in vivo*. Shown are confocal fluorescence lifetime images of CFP (A) and fluorescence lifetime distributions (B) in single living yeast cells expressing Pol30-CFPp (top), Pol30-CFPp plus Rtt109-YFPp (middle), and Pol30-CFPp plus Sas4-YFPp (bottom). Scale bar: 15 μ m.

TABLE 5
PCNA interacts with Rtt109p and SAS-I by FLIM-FRET analysis

Genotype	Lifetime	FRET efficiency	Distance
<i>ns</i>			
<i>POL30-CFP</i>	1.456 ± 0.134		<i>nm</i>
<i>POL30-CFP RTT109-YFP</i>	1.039 ± 0.161	0.286	6.055
<i>POL30-CFP SAS4-YFP</i>	1.012 ± 0.149	0.257	6.207

TABLE 6
pol30p mutants have defects in interacting with Rtt109p and SAS-I by FLIM-FRET analysis

Genotype	Lifetime	FRET efficiency
<i>ns</i>		
<i>POL30-CFP</i>	1.39 ± 0.0680	
<i>POL30-CFP SAS5-YFP</i>	1.11 ± 0.0241	0.201
<i>pol30-6-CFP SAS5-YFP</i>	1.41 ± 0.0901	
<i>pol30-8-CFP SAS5-YFP</i>	1.43 ± 0.0351	
<i>pol30-79-CFP SAS5-YFP</i>	ND ^a	ND
<i>pol30-6-CFP RTT109-YFP</i>	1.40 ± 0.0532	
<i>pol30-8-CFP RTT109-YFP</i>	1.39 ± 0.0636	
<i>pol30-79-CFP RTT109-YFP</i>	ND	ND

^a ND, not determined; lethal.

In contrast, no FRET was observed between Rtt109-YFPp and *pol30-8-* or *pol30-6-*CFPp; FRET interactions were also lost between SAS5-YFPp and *pol30-8-* or *pol30-6-*CFPp (Table

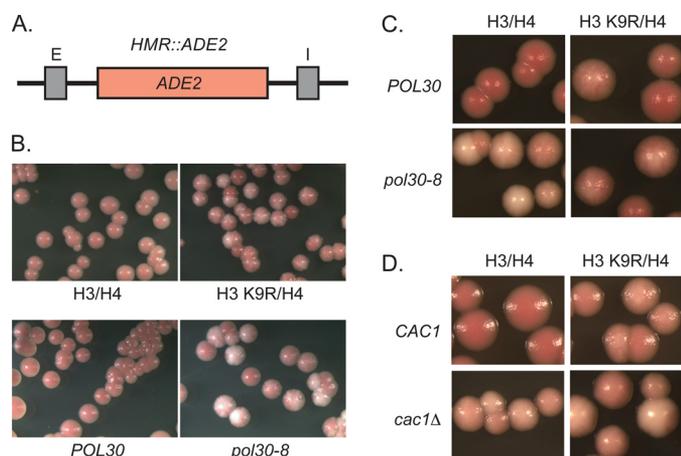


FIGURE 4. **POL30-dependent histone modifications influence silencing.** A, map of *HMR::ADE2*. B and C, overlapping silencing defects in histone H3 K9R and *pol30-8* mutants. D, overlapping silencing defects in histone H3 K9R and *cac1Δ* double mutants. Colony color assays were used to monitor silencing of the *ADE2* gene integrated at *HMR*. Cells were grown logarithmically, plated onto rich medium (YPD), incubated at 30 °C for 2 days, and then stored at 4 °C for 3 days prior to acquiring images (see “Experimental Procedures”).

6 and Fig. 6). FRET between *pol30-79*-CFPp and *Rtt109*-YFPp or *SAS5*-YFPp could not be assessed, as *POL30* plasmids could not be lost from strains co-expressing *pol30-79*-CFPp and *Rtt109*-YFPp or *SAS5*-YFPp during strain construction, indicating that these combinations may be lethal. These observations when combined support a model in which histone acetylation was coupled to PCNA and *pol30* mutants with defects in *ASF1*- and *CAC1*-dependent pathways had reduced levels of chromatin assembly factor-dependent histone modifications, which, in turn, affected silencing.

Altered Histone Modifications in *pol30* Mutants Affect Silencing—To assess further the relationship among PCNA, histone acetylation, and silencing, we compared the silencing of *pol30* and histone hypoacetylation mutants in two additional assays: silencing of an *ADE2* reporter gene integrated at *HMR* and silencing of *HMRae*** (Figs. 4 and 5, Table 7, and supplemental Table 6). We generated *HMR::ADE2* yeast expressing histone H3 or H4 mutants that mimicked the unacetylated forms of Lys-9 on H3 or Lys-16 on H4, H3 K9R and H4 K16R, and compared silencing in these mutants with *pol30* mutants using a colony color assay. Cells expressing histone H3 K9R phenocopied the silencing defect of *pol30-8* mutants at *HMR::ADE2* (Fig. 4, A and B). Sectorial colonies were observed in both histone H3 K9R and *pol30-8* mutants, whereas red colonies were formed by wild-type cells. Similarly, sectorial colonies were observed in *pol30-8* cells expressing histone H3 K9R (Fig. 4C). As *pol30-8* mutants primarily have silencing defects in a *CAC1*-dependent pathway (20, 38), we also examined the interactions between *cac1Δ* and histone H3 K9R at *HMR::ADE2* (Fig. 4D). Colonies of cells lacking *CAC1* and expressing wild-type histones H3 and H4 ranged from light pink to nearly white with occasional light pink and white sectors (Fig. 4D). Similar colony colors were observed in *cac1Δ* cells expressing histone H3 K9R. Double mutant analyses could not be conducted with H4 K16R and *pol30* mutants, as colonies of *HMR::ADE2* cells expressing histone H4 K16R were white and, therefore, derepressed (data not shown). In control experi-

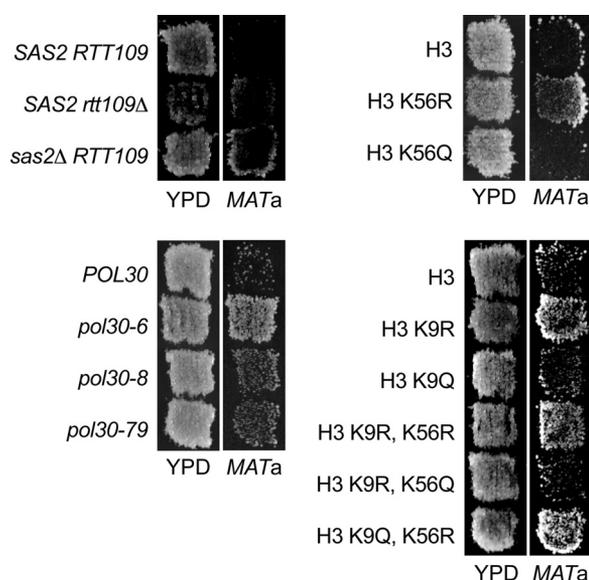


FIGURE 5. **Loss of H3-K9 acetylation restores silencing at *HMRae***.** *MATα HMRae*** strains expressing the indicated histone H3 mutants were grown on minimal medium with supplements for 24 h at 30 °C and then replicated to rich medium (YPD) or to *MATα* lawns (JRY2726) on minimal medium and incubated for 2 days at 30 °C. Restoration of silencing prevents expression of *a1* from *HMRae***, thereby enabling the *MATα* cells to mate and grow as diploids on minimal medium.

TABLE 7
Hypoacetylated histone mutants rescue silencing at *HMRae***

Histones	<i>HMR</i>	Relative efficiency of mating ^a
H3/H4	<i>HMR</i>	1
H3 K9R, K14R/H4 K16R	<i>HMR</i>	0.73 ± 0.11
H3/H4	<i>HMRae**</i>	0.011 ± 0.0051
H3 K9R/H4	<i>HMRae**</i>	0.067 ± 0.032
H3 K14R/H4	<i>HMRae**</i>	0.074 ± 0.055
H3/H4 K16R	<i>HMRae**</i>	0.070 ± 0.034
H3 K14R/H4 K16R	<i>HMRae**</i>	0.074 ± 0.060
H3 K9R, K14R/H4	<i>HMRae**</i>	0.20 ± 0.12
H3 K9R, K14R/H4 K16R	<i>HMRae**</i>	0.16 ± 0.15

^a The efficiency of mating of yeast containing *MATα HMR* plus wild-type histones H3 and H4 to tester strain JRY2726 (*MATα*) was determined relative to their plating efficiency on minimal (YM) medium containing supplements (45 ± 9.4%, *n* = 3) and was set to 1. The mating efficiency of each strain relative to *MATα HMR* is shown. Mean ± S.D., *n* = 3. Combining single mutations with each other correlated with enhanced silencing at *HMRae*** (*p* = 0.067; Jonckheere-Terpstra trend test).

ments, *ade2-1* strains containing a wild-type *HMR* locus plus *pol30-8*, *cac1Δ*, H3 K9R, or H4 K16R mutants all grew as red colonies (data not shown), confirming that these mutants did not disrupt the adenine metabolic pathway upstream of *ADE2*, which would have prevented the formation of a red pigment in a silencing-independent manner. The similar decrease of chromatin-associated H3-K9ac and the genetic epistasis among *pol30-8*, *cac1*, and H3 K9R suggest a network in which the PCNA-CAF-1 interaction ensures the deposition of H3-K9ac histones that contribute to silencing.

*HMRae*** can be used as a model locus for analyzing mutations that lead to the relocalization of Sir proteins to inappropriate genomic loci via defects in histone modifications (40, 79, 96). Sir proteins are not normally present at *HMRae*** because neither the mutated *E* silencer, which has a point mutation at its Rap1p binding site and one base pair insertion at its Abf1p binding site (100), nor the *I* silencer is capable of recruiting Sir proteins to *HMRae*** (9, 40). This lack of Sir binding is the cause

POL30 and the Establishment of Silencing

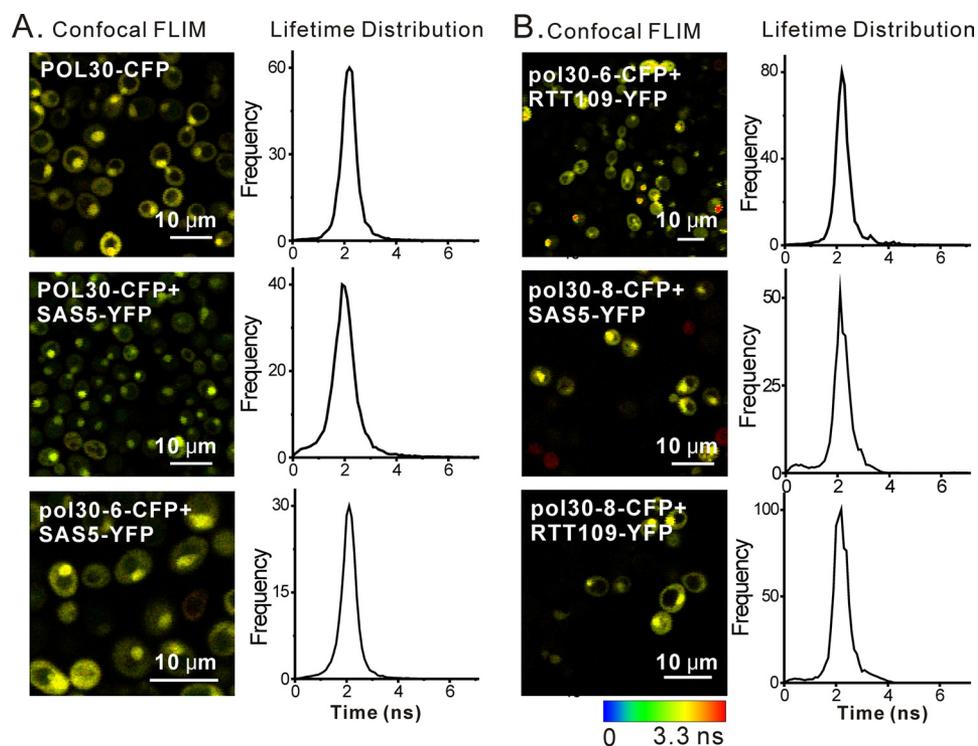


FIGURE 6. **pol30p mutants have defects in interacting with SAS-I and Rtt109p.** Interactions between pol30-CFPp mutants and Sas5-YFPp (A) or Rtt109-YFPp (B) are shown: confocal fluorescence lifetime image of CFP (left) and fluorescence lifetime distribution (right) in yeast expressing the indicated proteins. Scale bar: 10 μ m.

of the defect in silencing at this locus. In cells with hypoacetylated histones, however, Sir proteins become “mislocalized” to *HMRae*** via the presence of Sir binding sites on nucleosomes, thereby restoring silencing (e.g. see Refs. 40 and 79). Silencing at *HMRae*** is rescued in cells lacking *ASF1* or *CAC1* and in *pol30* mutants (19, 40, 96) (Fig. 5 and see below), implying that loss of chromatin assembly factor-dependent histone modifications had restored silencing in these strains. Consistent with this model, silencing defects at *HMRae*** are also partially suppressed in cells lacking *SAS2*, *SAS4*, or *SAS5* or expressing histone H4 K16R as well as in cells lacking *RTT109* or expressing H3 K56R (40, 96, 101–103) (Fig. 5). As Rtt109p acetylates Lys-9 in addition to Lys-56 on H3 (75, 84, 85, 104), we examined whether the loss of acetylation at Lys-9 on histone H3 could similarly suppress silencing defects at *HMRae*** and thereby restore mating in *MAT α* cells. In patch mating assays, silencing was rescued at *HMRae*** in H3 K9R mutants (Fig. 5). In contrast, *HMRae*** was derepressed in cells expressing H3 K9Q mutants. *HMRae*** was also derepressed in H3 K9R,K56Q mutants, but *HMRae*** became silenced in H3 K9R,K56R mutants as well as in H3 K9Q,K56R mutants. Thus, although H3 K9R could promote silencing at *HMRae***, the presence of a positive charge at residue 56 on H3 was more important than the charge status at residue 9 on H3 for silencing. In quantitative mating assays, H3 K9R mutants silenced *HMRae*** 6-fold more efficiently than did cells expressing wild-type histone H3 ($p = 0.018$, $n = 3$; Table 7).

We next tested for interactions between H3 K9R, H3 K14R and H4 K16R, as SAS-I readily acetylates both Lys-14 on H3 and Lys-16 on H4 but not Lys-9 on H3 *in vitro* (94). H3 K9R, H3 K14R, and H4 K16R mutants all restored silencing at *HMRae***

with similar efficiency, and combining H3 K14R with H4 K16R did not enhance silencing relative to either single mutant. In contrast, combining H3 K9R with H3 K14R and H4 K16R correlated with enhanced silencing at *HMRae*** (Table 7). Together, these results implied that loss of acetylation of Lys-9 on H3 restored silencing through a different pathway than did loss of acetylation of Lys-14 on H3 and Lys-16 on H4 and that H3-K14 and H4-K16 functioned through the same pathway. These pathways likely involve *RTT109* and *SAS2*, respectively, as well as *ASF1*, CAF-1, RF-C, and PCNA (see Fig. 6). Global hypoacetylation of histones in the *pol30* mutants from defects in replication-coupled chromatin assembly may have facilitated the mislocalization of Sir proteins to multiple genomic loci, thereby reducing the pools of Sir available for establishing silencing at chromosomal and extrachromosomal *HMRs* in earlier

experiments (Table 2). Consistent with this notion, cells expressing histones H3 K9,14R H4 K16R double mutants had mild defects in silencing at native *HM* loci as measured by quantitative mating assays (Table 7; see also Ref. 62), and cells lacking acetylation of these residues as well as of H3-K56 have severe silencing defects at *HML* (105).

To confirm that more than one pathway functioning through *POL30* was influencing silencing, we examined silencing at *HMRae*** in several *pol30* mutants. *pol30-79* plus *pol30-6* and *pol30-8* mutants have silencing defects that fall primarily in *ASF1*- or CAF-1-dependent pathways, respectively (20). *pol30-79* (Fig. 5), *pol30-6*, and *pol30-8* could each suppress the silencing defect at *HMRae*** ($p = 0.018$, $n = 3$ for each mutant), and *pol30-42*, which has the combined mutations of *pol30-6* and *pol30-8*, further enhanced silencing (Fig. 5 and supplemental Table 6). Other *pol30* mutants, which silence *HMR::ADE2* efficiently (data not shown) and have no reported defects in *ASF1*- or *CAC1*-dependent pathways, could not rescue silencing at *HMRae*** (supplemental Table 6). Together, these results implied that *pol30* mutants had restored silencing at *HMRae*** through multiple overlapping pathways involving *CAC1* and *ASF1*, which modulate histone acetylation (see also Ref. 40).

DISCUSSION

In *S. cerevisiae*, silencing at *HMR* can be established during the passage through S phase, yet the initiation of DNA replication at silencers and the passage of a replication fork through *HMR* are not required for silencing (10–13). Despite this lack of requirement for DNA replication in establishing silencing, this study has indicated that *pol30* mutants are defective in establishing silencing on both replicated and non-replicated *HMR*

loci upon passage through S phase (Table 2). These observations imply that defects in a replication-coupled role of PCNA led to global changes in chromatin that were incompatible with the formation of silent chromatin or that PCNA contributed to silencing directly in a locus-specific, replication-independent manner. However, as Pol30p functions in replication- and repair-coupled processes and Pol30p did not preferentially mark *HMR* prior to establishing silencing (supplemental Fig. 1), we favor the former possibility.

One replication-coupled way in which PCNA can influence silencing is through its role in directing the activity of chromatin assembly factors (20, 38). This role of PCNA connects DNA replication to nucleosome assembly and, hence, to the foundation upon which silenced chromatin is built as well as to chromatin at other loci across the genome. To understand how Pol30p influences chromatin structure and how chromatin structure impacts silencing, we analyzed the topology of *HMR* in *pol30* mutants with silencing defects. In this analysis, we separately assessed the influence of *pol30* mutants on chromatin structure in transcription-independent and Sir protein-dependent and -independent contexts. We reasoned that defects in packaging chromosomal DNA into nucleosomes after replication might lead to defects in silencing in the *pol30* mutants if the density of nucleosomes at *HMR* was insufficient to permit Sir spreading. Supporting this notion, DNA sequences that exclude nucleosome formation can act as a barrier to Sir spreading (106), and as shown by ChIP analyses, the level of histone H3 at *HMR* and other genomic loci is reduced in *cac1* mutants (76). As packaging DNA around nucleosomes induces negative supercoiling (107), gross defects in nucleosome density should have significantly altered the topology of *HMR* in *pol30* mutants in our experiments. If such defects in packaging DNA also prevented Sir association at *HMR*, then Sir-dependent changes in topology also should have been perturbed. Instead, we observed similar topological patterns in *pol30* versus *POL30* cells at a modified *HMR* lacking the *a1-a2* promoter region (supplemental Fig. 3). These findings revealed that mutant alleles of *POL30* did not lead to severe defects in packaging promoter-independent DNA into nucleosomes. Thus, *pol30* mutants likely compensated for defects in interacting with individual chromatin assembly factors by using alternative pathway(s) to ensure that the newly replicated DNA was packaged into chromatin. This compensation may have come at the cost, however, of incorporating inappropriately modified histones throughout the genome. We hypothesized that subtle *pol30*-dependent changes in the composition of chromatin, in turn, led to the defects in forming silent chromatin.

Under certain conditions, the rate of nucleosome assembly during DNA replication, rather than the final nucleosome density, could theoretically influence the establishment of silencing on a template that is replicated if a reduced rate of assembly resulted in the temporary loss of Sir protein binding sites on nucleosomes throughout *HMR*. Consistent with this model, loss of Asf1-dependent chromatin assembly in mammals results in delayed incorporation of histone H3 into chromatin (108, 109). If the rate of nucleosome assembly during DNA replication were significantly reduced in *pol30* mutants versus *POL30* cells, such a replication-coupled delay might lead to a

more severe defect in establishing silencing on a replicated versus non-replicated *HMR* locus during S phase. Instead, we found that *pol30-8* and *pol30-79* mutants had similar defects in establishing silencing on both replicated and non-replicated templates (Table 2). Because other pathways can take over chromatin assembly in the absence of Asf1p function (108), and modifications like H3-K56ac may influence nucleosome turnover (109, 110), it is still an open question as to whether the rate of chromatin assembly contributes to defects in silencing.

Coupling PCNA and Chromatin Assembly to Histone Modifications—Our analyses demonstrated that *pol30* mutants influenced histone modifications linked to chromatin assembly factors and that defects in these modifications altered silencing (Tables 3, 4, and 7, supplemental Fig. 4, Figs. 4 and 5, and Ref. 40). This study supports a central role for Pol30p in determining histone modifications in yeast.

Acetylation of Lys-9 on histone H3 is associated with newly synthesized histones (80, 81); this S phase-specific acetylation event likely depends on Asf1p and the histone acetyltransferases Rtt109p and Gcn5p (82–85) (see also Ref. 80). Our data imply that *CAC1* also plays a role in enabling acetylation of this residue. Consistent with this notion, acetylation of Lys-9 on histone H3 and the silencing defects of *pol30-8* and histone H3 K9R mutants correlate with a CAF-1-dependent pathway (Table 3 and Fig. 4) (20, 38). Our data also indicate that other histone modifications linked to *CAC1* or *ASF1* were perturbed in *pol30* mutants including SAS-I-dependent acetylation of Lys-16 on histone H4 (Table 4 and supplemental Fig. 4) and Rtt109p-dependent acetylation of Lys-56 on histone H3 (40). These findings imply that PCNA can influence silencing by affecting the levels of multiple modifications on histones in yeast.

The work presented here supports a model in which the differing defects in silencing in the various *pol30* mutants (19, 20, 38, 39), and likely other replication factors, are caused by defects in recruiting chromatin assembly factors to chromatin. This, in turn, leads to the incorporation of inappropriately modified histones throughout the genome. Hypoacetylated histones could help create high affinity binding sites for Sir proteins at multiple inappropriate loci across the genome (*HMRae*** being a model for one such locus) and result in an insufficient pool of Sir proteins being available for silent chromatin formation on replicated and non-replicated templates. Altered histone modifications at *HMR* initiated by errors in replication-coupled chromatin assembly during a previous S phase could also contribute to defects in establishing silencing. Future ChIP analyses will clarify how *pol30* and other replication fork mutants affect histone modifications genome-wide.

At least two possible mechanisms could account for how *POL30* affects histone modifications. First, in wild-type cells, newly synthesized histones could be post-translationally modified at a key residue(s) by a histone-modifying enzyme(s) prior to being loaded onto the chromosome during DNA replication, as has been suggested previously for H3-K9ac and -K56ac by Rtt109p (Refs. 75, 85, 87, and 111 and references therein). In this scenario, during replication, *pol30* mutants would have been defective in recruiting the correct classes of chromatin assembly factors, which were bound to correctly modified his-

POL30 and the Establishment of Silencing

tones. This defect would have led to the failure to load those histones onto the chromosome and to defects in silencing. The second possibility is that altered histone modifications in the *pol30* mutants were caused by a defect in targeting a key enzyme(s) to chromatin during or shortly after nucleosome assembly, analogous to replication-coupled modifications observed in mammals (see the Introduction). Our demonstration, using single molecule methods, that Rtt109p and SAS-I interact with PCNA *in vivo* and that these interactions are defective in the *pol30* mutants provides strong evidence for the second scenario but does not exclude the first possibility from occurring as well. Regardless of the mechanism(s) involved, the end result would be a genome with altered patterns of modifications on histones with the potential to negatively impact the regulation of individual genomic loci. Determining whether PCNA and chromatin assembly factors directly target histone acetyltransferases to the replication fork will help to clarify when and how these modifications occur. Currently, it also remains an open question as to whether or how specific chromatin assembly factors preferentially load different histones onto discrete regions of the genome during replication, but the targeted assembly of at least one variant, H2A.Z, is clearly influenced by modifications on histones H3 and H4 (112, 113). Further analysis of where specific histone modifications or, in more complex eukaryotes, histone variants linked to individual chromatin assembly factors are first observed throughout the genome upon DNA replication should begin to address this question (see (Refs. 114 and 115)).

Replication and the Establishment of Epigenetic Processes—Other evidence for replication-coupled events influencing the establishment of silencing in yeast has come from studies of cells lacking *SIR1*. In a given population of cells lacking *SIR1*, individual cells will exist in two distinct transcriptional states at the *HM* loci that are mitotically stable (116, 117). When changes in the transcriptional state are monitored in the progeny of a single derepressed *sir1Δ* cell by pedigree analysis, a phenomenon known as the “grandmother effect” can be observed (117). In these switching events, all “granddaughter” cells of the original derepressed cell will switch to the silenced state during the same generation, suggesting that an event linked to DNA replication in an earlier cell cycle was inherited in the progeny, which permitted the establishment of silencing during the following cell cycle. It is tempting to surmise that heritable histone modification states regulated by DNA replication might influence the probability of establishing silencing and be responsible for this grandmother effect. Such an event could include a replication-coupled modification critical for silencing or a modification that must be erased in a replication-dependent manner for silencing, as has been proposed for the loss of methylation on histone H3 during silent chromatin formation (118). Consistent with this, pedigree analyses indicate loss of *SET1*- or *DOT1*-dependent methylation of histone H3-K4 or H3-K9, respectively, increases the probability of establishing silencing in a given cell division whereas loss of *SAS2* decreases the likelihood that silencing will be established each cell cycle (119), analogous to our observations for *pol30* mutants (Table 2).

Summary—Taken together, the results presented here imply that mutations in PCNA can lead to alterations in histone modifications that influence the formation of epigenetic processes. Although advances have recently been made in deciphering chromatin modifications involved in transcription and the enzymes responsible for those modifications, the role of DNA replication in defining histone modifications throughout the genome is still poorly understood. Future studies examining the relationship between histone-modifying enzymes and factors at the DNA replication fork will provide insights into how chromatin structures at individual loci are maintained across generations.

Acknowledgments—We thank Scott Briggs, Peter Burgers, Ann Ehrenhofer-Murray, Marc Gartenberg, Jasper Rine, Mitch Smith, Sharon Roth, and Arnold Stein for protocols, reagents, strains, and plasmids. We also thank Bruce Craig and Alexander Lipka for assistance with statistical analyses, Bo Yang for technical assistance, and Joe Ogas and Jasper Rine for helpful discussions.

REFERENCES

1. Rusché, L. N., Kirchmaier, A. L., and Rine, J. (2003) *Annu. Rev. Biochem.* **72**, 481–516
2. Moretti, P., Freeman, K., Coodly, L., and Shore, D. (1994) *Genes Dev.* **8**, 2257–2269
3. Gardner, K. A., Rine, J., and Fox, C. A. (1999) *Genetics* **151**, 31–44
4. Triolo, T., and Sternglanz, R. (1996) *Nature* **381**, 251–253
5. Luo, K., Vega-Palas, M. A., and Grunstein, M. (2002) *Genes Dev.* **16**, 1528–1539
6. Bose, M. E., McConnell, K. H., Gardner-Aukema, K. A., Müller, U., Weinreich, M., Keck, J. L., and Fox, C. A. (2004) *Mol. Cell. Biol.* **24**, 774–786
7. Hoppe, G. J., Tanny, J. C., Rudner, A. D., Gerber, S. A., Danaie, S., Gygi, S. P., and Moazed, D. (2002) *Mol. Cell. Biol.* **22**, 4167–4180
8. Rudner, A. D., Hall, B. E., Ellenberger, T., and Moazed, D. (2005) *Mol. Cell. Biol.* **25**, 4514–4528
9. Rusché, L. N., Kirchmaier, A. L., and Rine, J. (2002) *Mol. Biol. Cell* **13**, 2207–2222
10. Fox, C. A., Ehrenhofer-Murray, A. E., Loo, S., and Rine, J. (1997) *Science* **276**, 1547–1551
11. Kirchmaier, A. L., and Rine, J. (2001) *Science* **291**, 646–650
12. Li, Y. C., Cheng, T. H., and Gartenberg, M. R. (2001) *Science* **291**, 650–653
13. Miller, A. M., and Nasmyth, K. A. (1984) *Nature* **312**, 247–251
14. Lau, A., Blitzblau, H., and Bell, S. P. (2002) *Genes Dev.* **16**, 2935–2945
15. Kaufman, P. D., Kobayashi, R., and Stillman, B. (1997) *Genes Dev.* **11**, 345–357
16. Enomoto, S., and Berman, J. (1998) *Genes Dev.* **12**, 219–232
17. Smith, J. S., Caputo, E., and Boeke, J. D. (1999) *Mol. Cell. Biol.* **19**, 3184–3197
18. Singer, M. S., Kahana, A., Wolf, A. J., Meisinger, L. L., Peterson, S. E., Goggin, C., Mahowald, M., and Gottschling, D. E. (1998) *Genetics* **150**, 613–632
19. Ehrenhofer-Murray, A. E., Kamakaka, R. T., and Rine, J. (1999) *Genetics* **153**, 1171–1182
20. Zhang, Z., Shibahara, K., and Stillman, B. (2000) *Nature* **408**, 221–225
21. Foss, M., McNally, F. J., Laurensen, P., and Rine, J. (1993) *Science* **262**, 1838–1844
22. Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B., and Stillman, B. (1987) *Nature* **326**, 471–475
23. Tan, C. K., Castillo, C., So, A. G., and Downey, K. M. (1986) *J. Biol. Chem.* **261**, 12310–12316
24. Maga, G., and Hübscher, U. (1995) *Biochemistry* **34**, 891–901
25. Hübscher, U., Maga, G., and Podust, V. N. (1996) in *DNA Replication in*

- Eukaryotic Cells* (DePamphilis, M. L., ed) pp. 525–543, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
26. Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) *Cell* **79**, 1233–1243
 27. Fien, K., and Stillman, B. (1992) *Mol. Cell. Biol.* **12**, 155–163
 28. Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutillier, K., Yang, L., Wolting, C., Donaldson, I., Schandorff, S., Shewnarane, J., Vo, M., Taggart, J., Goudreau, M., Muskut, B., Alfarano, C., Dewar, D., Lin, Z., Michalickova, K., Willems, A. R., Sassi, H., Nielsen, P. A., Rasmussen, K. J., Andersen, J. R., Johansen, L. E., Hansen, L. H., Jespersen, H., Podtelejnikov, A., Nielsen, E., Crawford, J., Poulsen, V., Sorensen, B. D., Matthiesen, J., Hendrickson, R. C., Gleeson, F., Pawson, T., Moran, M. F., Durocher, D., Mann, M., Hogue, C. W., Figeys, D., and Tyers, M. (2002) *Nature* **415**, 180–183
 29. Li, X., Li, J., Harrington, J., Lieber, M. R., and Burgers, P. M. (1995) *J. Biol. Chem.* **270**, 22109–22112
 30. Moldovan, G. L., Pfander, B., and Jentsch, S. (2007) *Cell* **129**, 665–679
 31. Burgers, P. M. (2009) *J. Biol. Chem.* **284**, 4041–4045
 32. Krawitz, D. C., Kama, T., and Kaufman, P. D. (2002) *Mol. Cell. Biol.* **22**, 614–625
 33. Shibahara, K., and Stillman, B. (1999) *Cell* **96**, 575–585
 34. Moggs, J. G., Grandi, P., Quivy, J. P., Jónsson, Z. O., Hübscher, U., Becker, P. B., and Almouzni, G. (2000) *Mol. Cell. Biol.* **20**, 1206–1218
 35. Kamakaka, R. T., Bulger, M., Kaufman, P. D., Stillman, B., and Kadonaga, J. T. (1996) *Mol. Cell. Biol.* **16**, 810–817
 36. Gaillard, P. H., Martini, E. M., Kaufman, P. D., Stillman, B., Moustacchi, E., and Almouzni, G. (1996) *Cell* **86**, 887–896
 37. Mello, J. A., Silljé, H. H., Roche, D. M., Kirschner, D. B., Nigg, E. A., and Almouzni, G. (2002) *EMBO Rep.* **3**, 329–334
 38. Sharp, J. A., Fouts, E. T., Krawitz, D. C., and Kaufman, P. D. (2001) *Curr. Biol.* **11**, 463–473
 39. Huang, S., Zhou, H., Katzmann, D., Hochstrasser, M., Atanasova, E., and Zhang, Z. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102**, 13410–13415
 40. Miller, A., Yang, B., Foster, T., and Kirchmaier, A. L. (2008) *Genetics* **179**, 793–809
 41. Franco, A. A., Lam, W. M., Burgers, P. M., and Kaufman, P. D. (2005) *Genes Dev.* **19**, 1365–1375
 42. Tyler, J. K., Collins, K. A., Prasad-Sinha, J., Amiot, E., Bulger, M., Harte, P. J., Kobayashi, R., and Kadonaga, J. T. (2001) *Mol. Cell. Biol.* **21**, 6574–6584
 43. Tyler, J. K., Adams, C. R., Chen, S. R., Kobayashi, R., Kamakaka, R. T., and Kadonaga, J. T. (1999) *Nature* **402**, 555–560
 44. Groth, A., Corpet, A., Cook, A. J., Roche, D., Bartek, J., Lukas, J., and Almouzni, G. (2007) *Science* **318**, 1928–1931
 45. Henderson, D. S., Banga, S. S., Grigliatti, T. A., and Boyd, J. B. (1994) *EMBO J.* **13**, 1450–1459
 46. Chuang, L. S., Ian, H. I., Koh, T. W., Ng, H. H., Xu, G., and Li, B. F. (1997) *Science* **277**, 1996–2000
 47. Li, E., Beard, C., and Jaenisch, R. (1993) *Nature* **366**, 362–365
 48. Estève, P. O., Chin, H. G., Smallwood, A., Feehery, G. R., Gangisetty, O., Karpf, A. R., Carey, M. F., and Pradhan, S. (2006) *Genes Dev.* **20**, 3089–3103
 49. Iida, T., Suetake, I., Tajima, S., Morioka, H., Ohta, S., Obuse, C., and Tsurimoto, T. (2002) *Genes Cells* **7**, 997–1007
 50. Milutinovic, S., Zhuang, Q., and Szyf, M. (2002) *J. Biol. Chem.* **277**, 20974–20978
 51. Fuks, F., Burgers, W. A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. (2000) *Nat. Genet.* **24**, 88–91
 52. Sarraf, S. A., and Stancheva, I. (2004) *Mol. Cell* **15**, 595–605
 53. Huen, M. S., Sy, S. M., van Deursen, J. M., and Chen, J. (2008) *J. Biol. Chem.* **283**, 11073–11077
 54. Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinberg, D., and Jenuwein, T. (2004) *Genes Dev.* **18**, 1251–1262
 55. Adams, A., Gottschling, D. E., Kaiser, C. A., and Stearns, T. (1997) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Plainview, NY
 56. Stearns, T., Ma, H., and Botstein, D. (1990) *Methods Enzymol.* **185**, 280–297
 57. Goldstein, A. L., and McCusker, J. H. (1999) *Yeast* **15**, 1541–1553
 58. Wach, A., Brachat, A., Pöhlmann, R., and Philippsen, P. (1994) *Yeast* **10**, 1793–1808
 59. Zhang, W., Bone, J. R., Edmondson, D. G., Turner, B. M., and Roth, S. Y. (1998) *EMBO J.* **17**, 3155–3167
 60. Sheff, M. A., and Thorn, K. S. (2004) *Yeast* **21**, 661–670
 61. Kirchmaier, A. L., and Rine, J. (2006) *Mol. Cell. Biol.* **26**, 852–862
 62. Yang, B., and Kirchmaier, A. L. (2006) *Mol. Biol. Cell* **17**, 5287–5297
 63. Daganzo, S. M., Erzberger, J. P., Lam, W. M., Skordalakes, E., Zhang, R., Franco, A. A., Brill, S. J., Adams, P. D., Berger, J. M., and Kaufman, P. D. (2003) *Curr. Biol.* **13**, 2148–2158
 64. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990) *Nucleic Acids Res.* **18**, 3091–3092
 65. Vidi, P. A., Chen, J., Irudayaraj, J. M., and Watts, V. J. (2008) *FEBS Lett.* **582**, 3985–3990
 66. Varghese, L. T., Sinha, R. K., and Irudayaraj, J. (2008) *Anal. Chim. Acta* **625**, 103–109
 67. Siegel, R. M., Chan, F. K., Zacharias, D. A., Swofford, R., Holmes, K. L., Tsien, R. Y., and Lenardo, M. J. (2000) *Sci. STKE* **2000**, PL1
 68. van Leeuwen, F., and Gottschling, D. E. (2002) *Methods Enzymol.* **350**, 165–186
 69. Martins-Taylor, K., Dula, M. L., and Holmes, S. G. (2004) *Genetics* **168**, 65–75
 70. Matecic, M., Martins-Taylor, K., Hickman, M., Tanny, J., Moazed, D., and Holmes, S. G. (2006) *Genetics* **173**, 1939–1950
 71. Ayyagari, R., Impellizzeri, K. J., Yoder, B. L., Gary, S. L., and Burgers, P. M. (1995) *Mol. Cell. Biol.* **15**, 4420–4429
 72. Eissenberg, J. C., Ayyagari, R., Gomes, X. V., and Burgers, P. M. (1997) *Mol. Cell. Biol.* **17**, 6367–6378
 73. Miller, A. M. (1984) *EMBO J.* **3**, 1061–1065
 74. Li, Q., Fazly, A. M., Zhou, H., Huang, S., Zhang, Z., and Stillman, B. (2009) *PLoS Genet.* **5**, e1000684
 75. Tsubota, T., Berndsen, C. E., Erkmann, J. A., Smith, C. L., Yang, L., Freitas, M. A., Denu, J. M., and Kaufman, P. D. (2007) *Mol. Cell* **25**, 703–712
 76. Tamburini, B. A., Carson, J. J., Linger, J. G., and Tyler, J. K. (2006) *Genetics* **173**, 599–610
 77. van Leeuwen, F., Gafken, P. R., and Gottschling, D. E. (2002) *Cell* **109**, 745–756
 78. van Leeuwen, F., and Gottschling, D. E. (2002) *Curr. Opin. Cell Biol.* **14**, 756–762
 79. Yang, B., Britton, J., and Kirchmaier, A. L. (2008) *J. Mol. Biol.* **381**, 826–844
 80. Kuo, M. H., Brownell, J. E., Sobel, R. E., Ranalli, T. A., Cook, R. G., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996) *Nature* **383**, 269–272
 81. Sobel, R. E., Cook, R. G., Perry, C. A., Annunziato, A. T., and Allis, C. D. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1237–1241
 82. Adkins, M. W., Carson, J. J., English, C. M., Ramey, C. J., and Tyler, J. K. (2007) *J. Biol. Chem.* **282**, 1334–1340
 83. Adkins, M. W., and Tyler, J. K. (2004) *J. Biol. Chem.* **279**, 52069–52074
 84. Berndsen, C. E., Tsubota, T., Lindner, S. E., Lee, S., Holton, J. M., Kaufman, P. D., Keck, J. L., and Denu, J. M. (2008) *Nat. Struct. Mol. Biol.* **15**, 948–956
 85. Fillingham, J., Recht, J., Silva, A. C., Suter, B., Emili, A., Stagljar, I., Krogan, N. J., Allis, C. D., Keogh, M. C., and Greenblatt, J. F. (2008) *Mol. Cell. Biol.* **28**, 4342–4353
 86. Celic, I., Masumoto, H., Griffith, W. P., Meluh, P., Cotter, R. J., Boeke, J. D., and Verreault, A. (2006) *Curr. Biol.* **16**, 1280–1289
 87. Driscoll, R., Hudson, A., and Jackson, S. P. (2007) *Science* **315**, 649–652
 88. Han, J., Zhou, H., Horazdovsky, B., Zhang, K., Xu, R. M., and Zhang, Z. (2007) *Science* **315**, 653–655
 89. Maas, N. L., Miller, K. M., DeFazio, L. G., and Toczyski, D. P. (2006) *Mol. Cell* **23**, 109–119
 90. Masumoto, H., Hawke, D., Kobayashi, R., and Verreault, A. (2005) *Nature* **436**, 294–298
 91. Recht, J., Tsubota, T., Tanny, J. C., Diaz, R. L., Berger, J. M., Zhang, X., Garcia, B. A., Shabanowitz, J., Burlingame, A. L., Hunt, D. F., Kaufman, P. D., and Allis, C. D. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103**, 6988–6993
 92. Xu, E. Y., Bi, X., Holland, M. J., Gottschling, D. E., and Broach, J. R. (2005)

POL30 and the Establishment of Silencing

- Mol. Cell. Biol.* **25**, 1846–1859
93. Zhou, H., Madden, B. J., Muddiman, D. C., and Zhang, Z. (2006) *Biochemistry* **45**, 2852–2861
94. Sutton, A., Shia, W. J., Band, D., Kaufman, P. D., Osada, S., Workman, J. L., and Sternglanz, R. (2003) *J. Biol. Chem.* **278**, 16887–16892
95. Shia, W. J., Osada, S., Florens, L., Swanson, S. K., Washburn, M. P., and Workman, J. L. (2005) *J. Biol. Chem.* **280**, 11987–11994
96. Meijnsing, S. H., and Ehrenhofer-Murray, A. E. (2001) *Genes Dev.* **15**, 3169–3182
97. Osada, S., Sutton, A., Muster, N., Brown, C. E., Yates, J. R., 3rd, Sternglanz, R., and Workman, J. L. (2001) *Genes Dev.* **15**, 3155–3168
98. Sutton, A., Bucaria, J., Osley, M. A., and Sternglanz, R. (2001) *Genetics* **158**, 587–596
99. McAlear, M. A., Tuffo, K. M., and Holm, C. (1996) *Genetics* **142**, 65–78
100. Kimmerly, W., Buchman, A., Kornberg, R., and Rine, J. (1988) *EMBO J.* **7**, 2241–2253
101. Ehrenhofer-Murray, A. E., Rivier, D. H., and Rine, J. (1997) *Genetics* **145**, 923–934
102. Xu, E. Y., Kim, S., Replogle, K., Rine, J., and Rivier, D. H. (1999) *Genetics* **153**, 13–23
103. Reifnyder, C., Lowell, J., Clarke, A., and Pillus, L. (1997) *Nat. Genet.* **16**, 109
104. Han, J., Zhou, H., Li, Z., Xu, R. M., and Zhang, Z. (2007) *J. Biol. Chem.* **282**, 14158–14164
105. Yang, B., Miller, A., and Kirchmaier, A. L. (2008) *Mol. Biol. Cell* **19**, 4993–5005
106. Bi, X., Yu, Q., Sandmeier, J. J., and Zou, Y. (2004) *Mol. Cell. Biol.* **24**, 2118–2131
107. Wang, J. C. (1982) *Cell* **29**, 724–726
108. Galvani, A., Courbeyrette, R., Agez, M., Ochsenbein, F., Mann, C., and Thuret, J. Y. (2008) *Mol. Cell. Biol.* **28**, 3672–3685
109. Kaplan, T., Liu, C. L., Erkmann, J. A., Holik, J., Grunstein, M., Kaufman, P. D., Friedman, N., and Rando, O. J. (2008) *PLoS Genet.* **4**, e1000270
110. Li, Q., Zhou, H., Wurtele, H., Davies, B., Horazdovsky, B., Verreault, A., and Zhang, Z. (2008) *Cell* **134**, 244–255
111. Han, J., Zhou, H., Li, Z., Xu, R. M., and Zhang, Z. (2007) *J. Biol. Chem.* **282**, 28587–28596
112. Raisner, R. M., Hartley, P. D., Meneghini, M. D., Bao, M. Z., Liu, C. L., Schreiber, S. L., Rando, O. J., and Madhani, H. D. (2005) *Cell* **123**, 233–248
113. Shia, W. J., Li, B., and Workman, J. L. (2006) *Genes Dev.* **20**, 2507–2512
114. Nakatani, Y., Ray-Gallet, D., Quivy, J. P., Tagami, H., and Almouzni, G. (2004) *Cold Spring Harbor Symp. Quant. Biol.* **69**, 273–280
115. Loyola, A., Bonaldi, T., Roche, D., Imhof, A., and Almouzni, G. (2006) *Mol. Cell* **24**, 309–316
116. Xu, E. Y., Zawadzki, K. A., and Broach, J. R. (2006) *Mol. Cell* **23**, 219–229
117. Pillus, L., and Rine, J. (1989) *Cell* **59**, 637–647
118. Katan-Khaykovich, Y., and Struhl, K. (2005) *EMBO J.* **24**, 2138–2149
119. Osborne, E. A., Dudoit, S., and Rine, J. (2009) *Nat. Genet.* **41**, 800–806